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Positional cloning of *Lps*, and the general role of toll-like receptors in the innate immune response

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RESUME / SUMMARY

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In mice (and by inference, in all mammals), a single pathway exists to serve lipopolysaccharide (LPS) signal transduction, and as such, allelic mutations at a single locus entirely abolish responses to LPS in C3H/HeJ and C57BL/10ScCr mice. Positional cloning of this locus, known as *Lps*, revealed that mutations of the Toll-like receptor 4 gene *(Tlr4)* are responsible for endotoxin resistance. A quick succession of studies have shown Tlr4 to be the critical transmembrane component of the LPS signal transduction complex. As LPS sensing by Tlr4 depends on physical contact between the two molecules, Tlr4 is a direct interface with the microbial world. Eight other molecules with strong similarily to Tlr4 are presently known in mammals, and taking Tlr4 as a model, all may be guessed to participate in the early detection of invasive pathogens. Acting together, the Toll-like receptors may be assumed to present macrophages with a comprehensive "picture" of the micobial world, and thus comprise the principal sensing molecules utilized by cells of the innate immune system.

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INTRODUCTION

In the course of evolution, multicellular organisms have developed immune mechanisms that protect them from the predations of unicellular life forms. The most fundamental attribute of any immune system is its ability to distinguish self from non-self. Beyond this, an immune system must be able to recognize a wide diversity of would-be pathogens if it is to be effective. In general, an immune system has a "sensing" (afferent) component, by which the host is alerted to infection, and an "effector" (efferent) component, comprising the response to infection; otherwise, the system would need to be continuously active, with all the liabilities that might imply. Plants, invertebrates, and vertebrates have employed different evolutionary strategies in pursuit of systems that fulfill these essential requirements.

Vertebrates alone have evolved a system of "specific immunity" (also called "acquired immunity," or "adaptive immunity"), marked by genomic recombination which yields an almost limitless number of receptors to engage microbial antigens. The specific immune system exhibits "memory," in the sense that subsequent encounters with an antigen yield quicker and stronger responses. But with few exceptions, the acquired immune system is initially näive: we are not born with specific immunity to most microbes, beyond that passively conferred by the transfer of immunoglobulin *in utero*. Specific immunity depends upon lymphocytes, and the endpoint of an acquired immune response is the secretion of antibodies that bind, opsinize, and destroy pathogens, or the development of specific T cell clones that lyse infected cells.

Since the beginning of the 20th century, immunologists have been aware that a second, more ancient immune system operates in parallel with the specific immune system, acting in many ways to support it. The so-called "innate" immune system (also called the "natural" immune system) functions immediately, and requires no prior exposure to the pathogen. It is based upon phagocytic cells (in the main, macrophages and neutrophils), which not only phagocytose microbial pathogens, but also elaborate cytokines that orchestrate inflammation and are essential for the development of the specific immune response.

Arguably, the innate immune system is more important to survival than the specific immune system. Without lymphocytes, mammals may live for years; without neutrophils, a few weeks might be considered the limit. As such, it is perhaps ironic that less is known about how innate immune cells recognize infection than about how specific immune cells do so. Antibodies and T cell receptors are the underpinning of microbial sensing in lymphocytes. The molecular details of their formation have been elucidated in considerable detail. Yet there has been much mystery concerning the cellular receptors for microbes on macrophages or neutrophils. How do cells of the innate immune system recognize that microbes have breached the physical defenses of the host?

The present review describes the identification of the mammalian endotoxin receptor, key component in a system for the recognition of Gram-negative organisms. The discovery that this protein is a member of the Toll-like receptor family has illuminated the sensing mechanism used in the innate immune system of vertebrates.

THE LPS PROBLEM

As recently reviewed by Rietschel and Westphal [1], R. Pfeiffer was the first to realize that a relatively insoluble component of Gram-negative organisms was responsible for the induction of fever, shock, and organ injury. He coined the term "endotoxin" to describe this factor. Many years later, ultrastructural and bioanalytical studies revealed that the outer membrane of Gram negative bacteria is a lipid bilayer, the outer leaflet of which is composed chiefly of an amphiphilic molecule of variable structure, termed lipopolysaccharide, or LPS. LPS is the chief component of Pfeiffer's "endotoxin", and the terms have come to be used interchangeably. The toxic center of LPS is the lipid A moiety; the polysaccharide component has little or no pharmacologic effect.

While Gram-positive organisms (which lack LPS) can cause fever, shock and organ injury that are clinically indistinguishable from the same problems caused by Gram-negative organisms, the injection of LPS can, by itself, reproduce many of the derangements observed in authentic sepsis. LPS is, in fact, a more potent toxin than any component of Gram-positive bacteria, excluding exotoxins. It has long been believed that LPS is responsible for several of the worst consequences of Gram-negative infection. At the same time (see below), it has become clear

that timely recognition of LPS is important, in the sense that early recognition of a Gram-negative infection can permit the host to overcome it.

For all of these reasons, LPS has been widely used for the purpose of modeling infection or inflammation. Implicit in much of this work was the notion that LPS was, *par excellence*, a "non-specific" activator of the innate immune system: probably the most powerful activator known. But how did it work?

LPS ACTION: FROM THE GENERAL TO THE SPECIFIC

The earliest notions of bacterial pathogenesis held that soluble toxins released by bacteria caused untoward effects in the host. Though rather tightly associated with the bacterial cell, endotoxin was assumed to act in this fashion as well. As recently as the 1970s, it was thought that LPS might interact with cells throughout the body, intercalating into lipid bilayers in a relatively nonspecific fashion and modifying ion flux, or otherwise perturbing biological membrane function. This, at any rate, was a standard view offered in medical microbiology texts.

A remarkable breakthrough was the demonstration that the lethal effect of LPS is mediated by lymphoreticular cells, and particularly, by macrophages. Hence, mice that are genetically unresponsive to LPS (see below) are made sensitive by the adoptive transfer of hematopoietic precursor cells from LPS sensitive donors. Similarly, LPS sensitive mice are rendered resistant if their hematopoietic stem cells are replaced with precursors from genetically resistant animals [2]. And the adoptive transfer of macrophages alone could confer sensitivity to resistant mice [3]. Much of the toxicity of LPS is mediated by TNF, which is secreted in abundance following macrophage activation [4-6]. Hence, passive immunization against TNF substantially attenuates LPS toxicity [7].

The critical role of lymphoreticular cells and the specific importance of TNF in the LPS response provided a focus for further experimentation, but did not answer questions related to the nature of the LPS receptor. Did LPS intercalate into the plasma membrane of macrophages at random? Or was there a specific receptor? In 1990, the glycosylphosphatidylinositol (GPI)-linked plasma membrane protein CD14 was identified as a proximal LPS receptor on the surface of macrophages [8]. CD14 seemed to receive LPS *via* transfer from the plasma protein LBP (lipopolysaccharide binding protein). Thereon, a signal was initiated. CD14-deficient mice were notably LPS resistant [9], and overexpression of CD14 could enhance sensitivity to LPS *in vivo* [10]. But the lack of a cytoplasmic domain made it seem that CD14 was not likely to act as the transducer. Rather, a "co-receptor" for LPS signal transduction must exist. Standard biochemical approaches, transfection assays, and immunologic strategies were all employed to search for this co-receptor, but without success.

KEY INSIGHTS PROVIDED BY LPS-RESISTANT MICE

In 1965, Heppner and Weiss reported that mice of the C3H/HeJ substrain survived the injection of *Salmonella* LPS at doses lethal to most mice [11]. Sultzer enlarged on this discovery in 1968, noting that leukocytic exudative responses to LPS were absent in C3H/HeJ mice [12]. Ultimately, it became clear that all responses to LPS, including cytokine production, were markedly depressed or absent in C3H/HeJ mice. Quite apart from this, mice of the C57BL/10ScCr strain were found to be resistant to LPS in 1977 [13].

In both instances, resistance was ascribed to mutations affecting a single locus. The C3H/HeJ mutation, described as codominant, was mapped to mouse chromosome 4 in 1978 [14, 15]. The relevant locus was termed *Lps*, to denote its importance in LPS responses. The mutant allele in C3H/HeJ mice was named Lps^d. The C57BL/10ScCr mutation, never formally named but described as recessive, was shown to be allelic with Lps^d at approximately the same time [16], in that mice produced by crossing C3H/HeJ animals to C57BL/10ScCr animals were highly resistant to LPS.

Mutations at the *Lps* locus had an important impact in immunology long before the identity of the gene was determined. Though the phenotype of C3H/HeJ and C57BL/10ScCr mice is being reappraised in light of recent discoveries, only LPS responses seemed to be impaired in these animals. Moreover, the defect was a general one (responses to *all* enteric LPS preparations

seemed to be impaired). Hence Lps^d allele of C3H/HeJ mice (and control animals of the C3H/HeN or C3H/OuJ substrains) found practical application in the exclusion of LPS as a participating factor in many experiments that had nothing to do with endotoxin *per se*. To cite one example, LPS was used to induce the synthesis of leukocyte activating factor (LAF), subsequently known as interleukin-1 (IL-1). The assay of IL-1 depended upon a thymocyte proliferation assay, performed using thymocytes from C3H/HeJ mice to avoid direct induction of proliferation by LPS.

More relevant to the LPS story, the mere fact that a single mutation could abolish LPS responses suggested that a solitary pathway for LPS signal transduction was present in mice. In the wake of the identification of CD14 as a biologically relevant LPS receptor, it was logical to believe that *Lps* might indeed encode a coreceptor for LPS signal transduction, though many other hypothesis were also be entertained [17-19].

Furthermore, the net phenotypic effect of LPS unresponsiveness was of interest in a practical sense. On the one hand, it might have been believed that LPS resistance would promote survival in sepsis, given the likely pathogenic importance of LPS in the development of shock. On the other hand, if responses to LPS have a negative impact on survival, why would they be retained by natural selection? In fact, numerous studies revealed that susceptibility to Gram-negative infection is enhanced by mutations at the LPS locus. On this basis, timely recognition of LPS (that is, recognition at a point preceeding overwhelming multiplication of the pathogen) would seem to be important to survival of the organism, permitting an effective immune response to be mounted [20, 21].

FINE MAPPING OF *Lps* AND THE DISCOVERY OF *Tlr4* AS THE LPS TRANSDUCER

By 1996, Qureshi, *et al.* had mapped *Lps* on 1,345 meioses to a point between proximal (Ambp, CD30L, and Hxb) and distal (D4MIT178 and D4MIT7) markers on mouse chromosome 4 which were separated by a genomic sequence of large and indeterminate size [22]. However, the critical region was never thoroughly explored by these investigators, and the *Lps* locus itself eluded them.

The mutations at the *Lps* locus of C3H/HeJ and C57BL/10ScCr mice were reported in September of 1998 by Poltorak and colleagues, who had independently mapped the position of the Lps^d mutation through analysis of 2,093 meioses, all produced by backcrosses to *Mus musculus* strains. Confining the mutation to a point between two novel markers that they had identified on chromosome 4 (designated B and 83.3), Poltorak, *et al.* cloned all of the genomic DNA in the *Lps* critical region (which at 2.6 million base pairs in physical size was one of the largest contigs ever assembled and analyzed in pursuit of a mouse mutation). Through high-density sequencing, exon trapping, and direct selection they identified a single candidate gene within the *Lps* critical region [23]. This candidate, the Toll-like receptor 4 gene *(Tlr4)*, was found to be mutated in C3H/HeJ mice [24]. A base transversion (C => A) in the third exon of the gene caused the substitution of a histidine for an evolutionarily conserved proline at residue 712 of the Tlr4 protein (P712H). Hence, the cytoplasmic domain of Tlr4 was modified in such a way as to yield a dominant inhibitory effect on signal transduction. In mice of the C57BL/10ScCr strain, a *Tlr4* null allele was identified [24], corresponding to the deletion of 74,723 nucleotides, encompassing all three exons of the gene [25]. The lack of *Tlr4* thus corresponds to a strictly recessive lesion [16]: a single copy of the gene is sufficient to confer normal LPS responsiveness.

EVOLUTIONARY INFERENCES ABOUT INNATE IMMUNITY, AND THE IMMEDIATE IMPLICATIONS OF THE IDENTITY OF *Tlr4* AND *Lps*

As pointed out earlier, much is known about the receptors of specific immunity (immunoglobulins and T cell receptors). Far less has been known about the receptors of vertebrate innate immunity. In fact, for the most part, there were few indications as to what these receptors might be.

Insight into the nature of these receptors first came from analyses carried out in invertebrates, which display *only* innate immunity. Studying fungal infection in *Drosophila melanogaster*

(conferred by expression of the antifungal peptide drosomycin), LeMaitre and coworkers found in 1996 that mutations of Toll - a gene encoding a plasma membrane receptor previously known only to govern polarity of the embryo - would create a state of susceptibility in adult flies [26]. Likewise, mutations of Spätzle, the ligand for Toll, and mutations of Pelle, Tube, and Dif [27] (which encode proteins that transduce the Toll signal thus permitting drosomycin synthesis) would lead to susceptibility. Other Toll-like receptors have also been identified in flies. One (18-wheeler) was found to confer resistance to bacterial infections, triggering the synthesis of the antimicrobial peptide attacin [28]. In no case, however, was the microbial inducer identified as a molecular entity, nor was direct contact between the Toll-like receptor and the microbial inducer presumed to occur.

Half a billion years have passed since the common ancestor of flies and mammals lived on earth, and it was not at all obvious that the vertebrate innate immune system would bear any semblance of Toll-like receptors, nor that these receptors would function in a manner similar to those of *Drosophila.* The first evidence of Toll-like receptor molecules in mammals emerged in 1991. At that time, the IL-1 receptor was noted to be structurally related to Toll. Further, signal transduction from IL-1R was ultimately found to proceed via MyD88, IRAK, and NFkappaB to permit the synthesis of numerous cytokines important to the immune response (Figure 1). Each of these molecules has a homologous counterpart in the Toll signaling pathway. Moreover, IL-1 (analogous but not homologous to Spätzle) was induced in response to microbial stimuli, and acted to trigger the synthesis of antimicrobial proteins (such as IL-2 and IL-6; again, analogous but not homologous to drosomycin). Subsequently, the second IL-1R chain, and the IL-18 receptor chains were found to have cytoplasmic Toll-like domains.

While Toll and 18-wheeler have leucine-rich extracellular domains, the IL-1R and IL-18R ectodomains are based on Ig-like repeats. Hence, they are imperfect facsimiles of Toll. With expansion in the complexity of expressed sequence tag (EST) databases, it became possible to search for other mammalian proteins with Toll-like domains. In due course, six of these were cloned, and were dubbed "Toll-like receptors" (TLRs). As of this writing, the sequences of cDNAs encoding human TLRs 1 through 6 reside in Genbank. However, three other TLRs have been identified by genomic sequencing, and their cDNAs cloned (Du, Poltorak, and Beutler, unpublished data).

The first full-length human TLR cDNA to be cloned was, in fact, TLR4 (then known as h-Toll). Medzhitov, *et al.*, showed that TLR4 could signal to cause nuclear translocation of NF-kappaB [29], though its ligand and biological function remained unclear. The presumption that it was involved in innate immunity, and signaled the development of adaptive immunity was insightful, yet the question of what might trigger the receptor was left unresolved.

The realization that Tlr4 was encoded by the *Lps* gene cast the Tlr family of in a new light. Since mutations of Toll cause enhanced susceptibility to fungal infection in fruit flies, whereas mutations of Tlr4 cause enhanced susceptibility to Gram-negative infection in mice, it became clear that the Tlrs did, indeed, represent an evolutionarily conserved innate immune mechanism for the recognition of specific pathogen molecules. Moreover, the impressive specificity of Tlr4 (which was at that time known *only* to respond to LPS) suggested that there was probably "oligospecificity" of these receptors. Hence, a very limited number of receptors would provide coverage of the entire microbial world.

While Toll signaling is initiated by Spätzle rather than by direct contact with an inducer of fungal origin, the mammalian homolog Tlr4 signals following direct contact with LPS (see below). The proteolytic cascade that yields processed Spätzle required for dorsoventral polarization of the embryo in fruit flies seems to have been diverted to other purposes in vertebrates. This cascade begins with *Gd* (Gastrulation defective), which cleaves *Easter*; *Easter* in turn cleaves *Snake*; and *Snake* cleaves pro-Spätzle to yield Spätzle. The closest mammalian homolog of *Gd* is the key coagulation cascade component prothrombin; the closest mammalian homolog of Easter is complement component C1S; the closest homologs of Snake are clotting factors X and XI, kallikrein, and other secreted serine proteases. Notably, Snake is also similar to components of the *Limulus* amoebacyte coagulation cascade (particularly factors B and G), which is activated following exposure to LPS (Figure 2).

The nature of the cascade that is activated by infectious stimuli (*e.g.*, fungal infection) has not yet been elucidated, though a serpin inhibitor (Spn43Ac) blocking Spätzle cleavage in response to

infection has recently been identified [30]. Overall, it would appear that evolution has retained homologs of the proteases upstream of Spätzle for defensive and inflammatory purposes. Yet it is not clear that *any* product of the complement or coagulation cascades in mammals can trigger signaling *via* the LPS receptor, and in fact, there is good reason to believe that the opposite is true.

STUDIES OF TLR4 SIGNAL TRANSDUCTION IN MACROPHAGES

The identity of *Tlr4* and *Lps* was proven beyond doubt by genetic studies. There was, nonetheless, strong motivation for biological studies of Tlr4 signal transduction, and there were many questions to address concerning the mode of action of the receptor. Did Tlr4 signal alone, or as a complex with CD14? Was it multimeric, and if so, did it form a heteromer, perhaps with other Tlrs? Why did the P712H mutation confer a codominant effect? These and other questions have not been answered in full, but an approach to their resolution has been made based on transfection studies, carried out in macrophages. While overexpression of Tlr4 in other cells did not reveal its role in LPS signal transduction at all [31], macrophages are known to possess all of the proteins required for transduction of the LPS signal. The most relevant endpoint of LPS signal transduction in macrophages is the elicitation of TNF secretion. Hence, the macrophage system, and the endpoint of TNF production, were chosen for analysis.

Overexpression of Tlr4 was shown to markedly enhance LPS signal transduction in RAW 264.7 mouse macrophages, while overexpression of the $Tlr4^{Lps\text{-}d}$ mutant blocked signal transduction [32]. As such, it was shown that Tlr4 is a limiting factor in LPS signal transduction, though this does not discount the importance of upstream (CD14) and downstream (*e.g.*, MyD88) components of the transduction apparatus.

Interestingly, deletion of the Tlr4 cytoplasmic domain does not confer an inhibitory effect on signal transduction. On this basis, it may be concluded that there is something distinctive about the P712H mutation. Two hypotheses (neither yet established as correct) immediately present themselves. TheTlr4 protein may very well be multimeric (either heterodimeric or homodomeric). Starting from this assumption, it might be suggested that P712H does not disrupt multimer assembly, but does prevent signaling by the assembled multimer. It might further be offered that the cytoplasmic domain of Tlr4 may supply binding energy for the interaction of subunits. In this scenario, the lack of a cytoplasmic domain would lead to preferential association of subunits that have a normal cytoplasmic domain, and hence, to persistant signal transduction regardless of the quantity of the mutant protein expressed. As such, cytoplasmic domain deletion would have a recessive phenotype, while the P712H mutation would be codominant. On the other hand, the P712H mutation might actively "tie up" transduction factors essential for propagation of the LPS signal. Interestingly, overexpression of a membrane-anchored form of the normal Tlr4 cytoplasmic domain has a weak inhibitory effect on LPS signaling, while overexpression of the P712H mutant form of the Tlr4 cytoplasmic has a slightly stronger inhibitory effect. This fact might favor the latter hypothesis concerning the mode of action of Lps^d, but provides no definitive proof.

From the fact that overexpression of the Tlr4 ectodomain alone fails to block LPS signal transduction in macrophages [32], it may be inferred that soluble versions of Tlr4 are unlikely to have a major influence on LPS signal transduction *in vivo*.

The Lps^d allele could also be fully complemented in immortalized endotoxin-resistant macrophages derived from C3H/HeJ mice. In such cells [33], retroviral expression of the normal isoform of the protein led to responses equal to, or greater than those observed in wild-type macrophages. This complementation system provides a means of testing the phenotype of various human TLR4 mutations as well (see below).

A further wrinkle of complexity has been introduced in the Tlr4 story with the demonstration that MD-2, a secreted protein produced by macrophages and other cells, appears to be physically associated with TLR4 on the surface of human mononuclear cells. It has been suggested that MD-2 is the final "missing component" required for LPS signal transduction in human embryonic kidney (HEK) cells, wherein transfection-based expression of TLR4 alone is not sufficient for LPS signal transduction [34]. While the fact of MD-2 interaction with TLR4 gives reason to believe that the protein might be important in LPS signal transduction, the transfection-based approach to

detecting participants in the signaling cascade has been badly discredited by the spurious claim that TLR2 (and not TLR4) is the critical protein in LPS signal transduction [31,35]. This latter suggestion was overturned by studies in which the *Tlr2* gene was knocked out in mice, which nonetheless showed perfectly normal LPS signaling [36]. Predictably, knockouts of Tlr4 show no response to LPS [36, 37]. If MD-2 is indeed required for LPS signal transduction, a similar phenotype would be expected.

LPS IS A DIRECT LIGAND FOR TLR4

As the composite picture of LPS signal transduction was modified to include Tlr4, several possibilities presented themselves. Once immobilized on CD14, LPS might engage Tlr4 as part of a ternary complex, triggering activation. Alternatively, LPS might be transferred to Tlr4, much as it is transferred to CD14 through a catalytic effect of LBP [38-42]. Finally, a series of reactions - perhaps proteolytic as in the *Drosophila* model - might be interposed between CD14 and Tlr4. At present no clear distinction can be made between the first two models. The last hypothesis however, formally suggested by Wright [43], has been excluded by a genetic complementation study revealing physical interaction between Tlr4 and LPS [33]. In this study, advantage was taken of a well-known species difference in sensitivity to modified forms of lipid A. It was previously established that while murine cells respond to tetra-acyl lipid A, human cells are unable to do so. In fact, tetra-acyl lipid A antagonizes the effects of intact lipid A (which has one to three acyloxyacyl-linked secondary aliphatic chains) when mixtures of the two LPS partial structures are applied to LPS-responsive human cells. Using retrovirally transfected immortalized C3H/HeJ macrophages as the basis of an assay system in which transduction through Tlr4 could be specifically monitored, Poltorak, *et al.* observed that the species origin of Tlr4 would predict the response to tetra-acyl lipid A. Human (but not mouse) Tlr4 is capable of discriminating between lipid A and tetra-acyl lipid A. Since the human Tlr4 molecule can distinguish between the presence or absence of two acyl side groups, it may reasonably be concluded that the protein has direct contact with the agonist in the course of signal transduction (Figure 3). Concordant with this finding, hamster cells fail to recognize tetra-acyl lipid A when transfected to overexpress human Tlr4 [44].

The same experiment proves that a structural difference between Tlr4 molecules (in this case, the difference between human and mouse Tlr4) may have an important influence on the perception of a particular molecular form of LPS, while permitting roughly equal levels of signal transduction in response to other forms of LPS. The question then arises as to whether polymorphism of Tlr4 (see below) migh have an influence on the sensing of (and resistance to) particular Gram-negative pathogens in humans.

SIGNAL TRANSDUCTION INITIATED BY TLR4

Our understanding of the complete structure of the LPS activation complex is still quite skeletal. In addition to Tlr4 and CD14, it has, as mentioned above, been suggested that MD-2 may be required for LPS signaling [34], though this assertion has yet to survive rigorous analysis (*i.e.*, knockout studies). The juxtamembrane protein moesin has also been implicated in LPS signaling by antibody neutralization work [45]. Here too, firm establishment of any role awaits knockout of the moesin gene, either in mice or at least in cultured cells. The proposed role of CD11b and CD18 (*i.e.*, Mac-1 or CR3) in LPS signal transduction [46-49] is open to doubt on the grounds that deficiency states involving either protein, in humans or animals, are not marked by evidence that LPS signaling is impaired.

Beneath the plasma membrane, at least some of the critical transduction proteins have been identified. MyD88, a cytoplasmic protein with a carboxy-terminal Toll-like domain and amino-terminal death domains, is recruited to the receptor and is presumed to engage in heterotypic association with the Tlr4 Toll-like domain. The MyD88 signal is propagated through IRAK (which also has death domains, and which presumably associates with MyD88 on this basis), and through TRAF-6. ECSIT is a newly-discovered protein that forms a bridge between TRAF-6 and MEKK1 [50]. The latter phosphorylates IkappaB, and thereby permits NFkappaB translocation (Figure 4).

Knockout of *Tlr4* [37] selectively abolishes LPS signal transduction, phenotypically mimicking the natural *Tlr4* deletion mutation observed in C57BL/10ScCr mice. Knockout of MyD88 [51] greatly attenuates LPS signal transduction, though it does not abolish all aspects of signaling from the

Tlr4 receptor. For example, NFkappaB activation occurs, but after a longer period of time following stimulation with LPS. LPS-induced MAPK phosphorylation can also be observed in cells from such knockout animals. Moreover, MyD88 knockout is less selective, in that it creates a phenotype in which IL-1 and IL-18 signal transduction are impaired along with LPS signal transduction [52]. Hence, it may be concluded that MyD88 is essential for transduction of at least some signals from all three of these receptors, and possibly from all Toll-bearing receptors. It is likely that other proteins (perhaps homologs of MyD88 yet undiscovered) are also required for Tlr-initiated signal transduction. This would seem to be the case for three reasons. First, there are notable differences in signal transduction initiated by LPS, IL-1, and IL-18, suggesting that more than one transduction coupler must be at work. Second, some signals transduced by Tlr4 persist in the absence of MyD88. And third, a single-gene mutation can evidently block signal transduction *via* Tlr4 and IL-1R in humans [53]. However, a genetic aberration has yet to be found in the expression or structure of the MyD88 in the patient concerned.

It is worth pointing out that, in addition to activation of NF-kappaB translocation, LPS induces many other biochemical changes in macrophages. All emanate from Tlr4, as all are absent in the cells of C3H/HeJ mice. These include activation of the p38 pathway [54], implicated in translational derepression of the TNF message, activation of the classical MAP kinase pathway [55], the SAP/Jun kinase pathway [56], and the PI3 kinase pathway [57]. Hence many details of coupling remain to be deciphered.

THE TLR FAMILY AT PRESENT

With 80% of the human genome now sequenced, nine strong Tlr homologs are represented in the HTGS division of Genbank (Table 1). These include genes encoding Tlr representatives that were not identified as ESTs, or indeed, by any other means (Tlrs 7, 8 and 9 [60]). The complete genomic sequence of TLR4 has also been established, from both humans and mice [58].

Two phylogenetic divisions of TLRs are recognizable by sequence analysis. TLRs 7, 8, and 9 are most closely related to one another, both in terms of length (they are approximately 1,000 amino acids in length) and sequence homology. All other TLRs fall into the second group. Since no functional distinctions have yet been made between the two groups, there is no certaintly as to whether the phylogenetic separation is biologically useful (Figure 5).

Deletion of the Tlr2 gene through homologous recombination in mouse embryonic stem cells has established that it is required for peptidoglycan [36] and lipopeptide [59] sensing. Moreover, knockout mice lacking Tlr4 are reportedly insensitive to lipoteichoic acid [36], an observation that has been confirmed using C3H/HeJ and C3H/HeN animals (Poltorak and Beutler, unpublished observation). As such, each Tlr molecule may subserve the recognition of a discrete collection of microbial ligands with very different structures.

Although enteric LPS molecules signal only *via* Tlr4, it cannot be excluded that some LPS molecules make use of other receptors, neither can it be said at present whether heteromer formation occurs among Tlrs, yielding combinatorial amplification of the receptor repertoire. The epitopic requirements for binding remain unknown, as do details of conformational changes that occur with binding. Despite these gaps in understanding, it can now be declared that the number of Tlrs is relatively small (*i.e.*, on the order of 10 rather than 100 or more). It can also be said that the general strategy of the innate immune system seems to be one in which indispensable microbial components (like the lipid A moiety of LPS, and like peptidoglycan) are targeted for recognition. In this manner, with but a few receptors, most of the microbial world can be "seen."

In addition to the "true" Toll-like receptors, a certain cytokines appear to utilize receptors with Toll-like domains. Among these, as mentioned above, are IL-1 and IL-18. In addition, an orphan receptor (SIGIRR) has been identified, which likely engages a cytokine mediator yet to be identified. Unlike the TLRs, SIGIRR has a relatively small ectodomain lacking the leucine-rich repeats that typify the prototypic Toll protein.

Several mammalian proteins show fairly strong homology to the Tlr ectodomains, yet have no Toll-like cytoplasmic domain, nor, in some cases, any cytoplasmic domain at all. Among these are the insulin-like growth factor binding protein, and the platelet glycoprotein V. The ribonuclease inhibitor protein is a far shorter, cytoplasmic protein composed almost entirely of leucine-rich repeats. It would seem likely that the leucine-rich motif is suited to the construction of

binding domains under diverse circumstances, and that evolution seized upon its potential as a building block very long ago.

POLYMORPHISM AT THE TLR4 LOCUS

A still-undefined mutation, observed in a 15-year-old girl in 1997 by Kuhns, *et al.*, attests to the overall importance of Tlrs in human immunity. This mutation abolished responses to IL-1 and LPS, and perhaps other ligands that signal *via* Tlrs. Phenotypically, the patient was severely immunocompromised, and had experienced numerous life-threatening infections by both Gram-negative and Gram-positive bacteria [53]. Hence, the Tlrs are extremely important for timely responses to infectious organisms.

Presumably, mutational inactivation of a single Tlr would present a more subtle phenotype than mutational inactivation of several (or all) Tlrs. Yet the question arises: do polymorphisms among Tlrs themselves influence responses to specific microbial pathogens? Might an ectodomain mutation of TLR4, for example, make a person more resistant to some Gram-negative pathogens and less resistant to others? The question is all the more cogent given the fact that LPS has direct contact with Tlr4, and given the added fact that large mutational differences between Tlr4 molecules (such as the difference between human and mouse Tlr4) cause dramatic differences in the response to a specific LPS partial structure (tetra-acyl lipid A) [33].

In mice, the exon sequence of *Tlr4* has been established in 37 Mus musculus strains [58]. A considerable amount of coding sequence polymorphism is apparent, and haplotype analysis may be used to trace the ancestry of several of the mutant alleles. Although phenotypic correlates of the distinct Tlr4 isoforms expressed by these different strains have not yet been established, these studies lay the groundwork for future experiments that might establish whether differences in susceptibility to Gram-negative organisms may be traced to structural differences in the Tlr4 ectodomain.

In humans, pronounced inter-ethnic variation in TLR4 sequence has been identified, and may reflect differences in the selective pressures to which humans of different geographic origins were exposed. Among Caucasians, a double amino acid substitution (the TLR4-B allele) is the most common variant, present in approximately 12% of the population. This allele is rare among African Americans, suggesting a European origin. Individuals of African origin, however, often have the more proximal of the two mutations, implying that the European allele may have been preceded by an African mutation, and built upon it.

Although phenotypic associations with TLR4 mutations are presently lacking, as are surveys of the promoter region for polymorphism, it is possible that mutations of TLR4 affect reactivity to specific LPS structures. Moreover, it may be imagined that, as in the case of the *Drosophila* Toll gene, in which certain mutations confer gain of function, some mutations of TLR4 might cause hypersensitivity to all LPS molecules, to other molecules yet unknown, or for that matter, cause constitutive activation of the innate immune system.

CONCLUSION

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Positional cloning work has established that Tlr4 is the sole transducer of the signal initiated by enteric LPS. As such, it has become a paradigm for innate immune sensors in vertebrates. Unlike the situation with its homolog Toll in *Drosophila* which senses infection through the intermediary protein Spätzle, Tlr4 directly engages LPS. The future challenge in what now be called the "Tlr field" will be to identify which ligands activate each Tlr. When this has been accomplished, a good deal more will be known about the crucial interface between microbes and the cells that respond to them.

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